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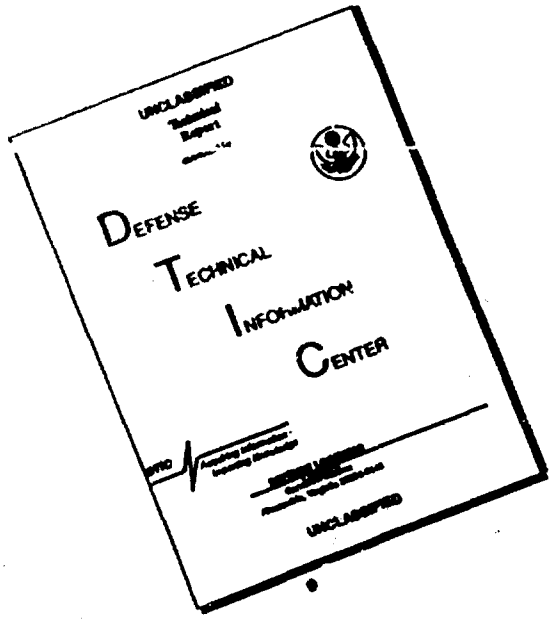

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THE USE OF INFRARED SPECTROPHOTOMETRY TO ACCELERATE THE IDENTIFICATION OF SANITARY-SIGNIFICATIVE MICROBES

[Following is the translation of an article by V. I. Bugrova, Moscow Scientific-Research Institute of Hygiene imeni F. F. Erisman, published in the Russian-language publication Vop. San. Bakt. Virus., 1965, pages 61--68.]

In recent years infrared spectrophotometric analysis has found wide application not only in physics, chemistry, and biochemistry, but also in microbiology. Due to the fact that an organic substance has its own inherent absorption spectrum in the infrared range of radiation, this property is used with success for a qualitative analysis of organic substances and for clearing up their molecular structure. Thus, for example, with the help of infrared spectrophotometric analysis Levine and associates already in 1955 established that absorption bands in the range between 6.4 and 6.5 microns are connected mainly with the polypeptide chain of protein, in the range from 8 to 8.1 microns with nucleic acids, and in the range from 8.6 to 10 microns with nucleic acids and carbohydrates simultaneously. Spectral differences are revealed easiest of all based on the contours of the last band.

Bands of 5.7 - 5.8 microns indicate an increased amount of fatty acids.

The majority of species and type biological properties of microorganisms are connected primarily with these substances and infrared spectrophotometric analysis makes it possible to study the composition of the microbial cell. At the basis of difference between microorganisms lies the difference in their chemical structure, therefore each species has its own more or less individual spectrum of absorption of infrared rays. This method was used for the identification of microbes for the first time in 1952 by Stiwenson and associates. At present foreign investigators have accumulated a considerable amount of material on infrared spectroscopy of microorganisms. The methodical work by Yu. S. Vaylya and V. M. Klevakin was published in our press in 1962. In spite of the fact that infrared spectroscopy of microorganisms has been treated in a relatively great number of foreign papers, the methodical problems of these investigations still cannot be considered resolved completely.

Using the contemporary IKS-14 infrared spectrophotometer we attempted to work out certain methodical problems in the accelerated determination of microbial species under the conditions of our own laboratories. As objects of investigation we selected sanitary-significative microbes - E. coli, enterococcus, and staphylococcus. Development of methods and the obtaining of spectrograms of these microbes were carried out using cultures which were obtained from the Control Institute imeni Tarasevich.

It is known from data in the literature that the nature of constancy of the spectrum of one and the same microorganism can be influenced by the nutrient medium on which they were incubated, duration and temperature of incubation, and also the thoroughness of calibration (adjustment) of devices used for their study. Therefore we considered it expedient to incubate all the cultures being studied on standard media (nutrient media of the same series of preparation and uniform in composition) while observing the same conditions of incubation.

For spectrophotometric study it is possible to use not only whole microbial cells, but also extracts obtained from them and containing the various chemical components of the cells.

We studied the spectra of whole microbial cells in preparations which were prepared from cultures which had been thoroughly washed with distilled water and those without washing; these were carefully removed from the surface of the nutrient media with a loop and placed on the plates.

There were no significant differences in the recordings of spectra of washed and unwashed cultures.

We also studied the spectra of extracts of the same cultures. For obtaining of extracts initially the microbial cells were broken down, then the destroyed cell mass was extracted with acetone and evaporated in order to obtain the concentrated extracts. These were placed on plates, dried, and studied. While for obtaining preparations from non-destroyed microbial cells the method was relatively simple, the obtaining of extracts was somewhat complicated by the necessity of breaking down the microbial cells in specially prepared homoganizers.

We placed the microbes and extracts on polished plates made from silver chloride and dried them in a thermostat. Plates made from silver chloride are most convenient since infrared rays pass through easily and they possess neglible solubility in water (0.00015 γ in 100 g of water).

The preparations were subjected to additional drying in the infrared rays. For obtaining objective data each preparation was recorded 2--3 times. Measurement of absorption spectrum of infrared rays of microbial cells and their extracts was carried out at wavelengths from 5 to 15 microns, which corresponds to the passage of infrared rays by a prism made from a single crystal of NaCl. At various wavelengths a more complete concept of the structure of microorganisms is obtained and differences between them are revealed more clearly.

Since we incubated the culture on dense nutrient media, then naturally at first we took the spectra of the nutrient media used in the work. E. coli were incubated on MPA /meat peptone agar/ and the enterococci and staphylococci on blood agar. The spectrograms of the nutrient media gave us a concept of their characteristic bands of absorption of infrared rays.

All the spectrograms of the cultures and extracts are presented on the charts, where along the axis of ordinates are given the percentages of absorption of infrared rays by the cultures, and along the axis of abscissae - wavelengths of transient rays in microns.

As can be seen from the drawings, the main picture of spectral absorption lies in the interval from 5 to 12 microns, more exactly 6.5, 8.1, and 10 microns for cultures of microbes, and 5.8, 6.5, 8.1, 9.2, 10, and 11.7 microns in spectrograms of extracts of these same cultures.

With a superficial acquaintance with the spectrograms of the various types of E. coli, enterococci, and staphylococci they all are as if similar, and only following an attentive study of them are specific differences apparent, often in details.

The picture of spectral absorption in the interval of wavelengths of 5--12 microns is generally complex, particularly in the spectrograms of extracts of microbial cells. In this interval the various extracted chemical components are recorded (polypeptides, amino acids, carbohydrates, fatty acids, etc.). The nature of the spectrum in this region is such that the recorded microbes and their extracts can be identified only by the direct matching of the analogous spectral curve. Differences between them were expressed more clearly in the last bands of the spectrogram.

In the upper left of Fig. 1 are depicted the three spectrograms of non-destroyed microbial cells: enterococci, E. coli, and staphylococci. The last two bands, lying in the region from 8 to 10 microns, are characteristic for these microbes. The spectrograms differ only in their configuration. These bands, according to data from the literature, should correspond to nucleic acids and carbohydrates. The 6.5 micron band indicates the presence of protein polypeptide chains.

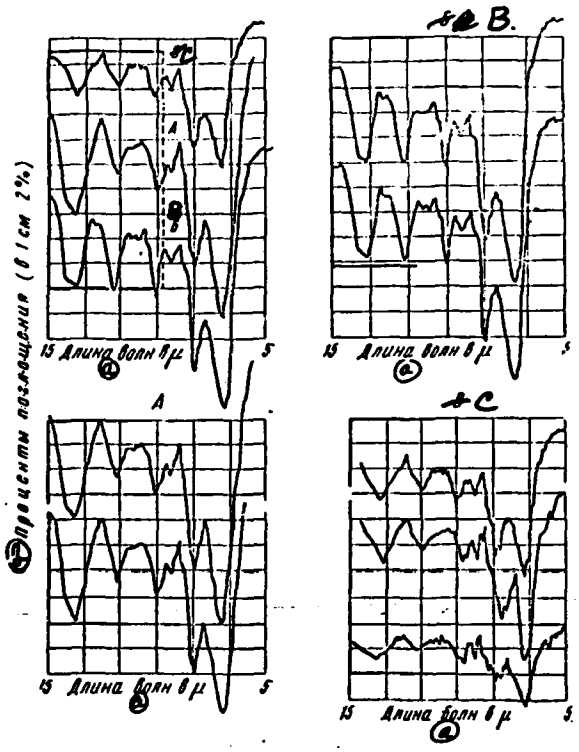

Fig. 1. Spectra of cultures: A - E. coli; B - staphylococci; C - enterococci.
Key: (a) Wavelength in microns; (b) Percentages of absorption (in 1 cm 2%).

On the individual spectrograms, designated by the letters A, B, and C, paired curves are given which show the recurrence of the recording of the same cultures, in this case A - E. coli, B - staphylococci, and C - enterococci. The insignificant differences which can be seen in these spectrograms lie within the limits of error of the device. The grid with the spectrograms of enterococci contains a third curve, recorded with a very thin stroke; basically it has the same nature as the two located above, the same wavelengths in the characteristic bands. This indicates that in spite of quantitative differences, caused by the thickness of strokes, the microorganisms can still be differentiated based on the nature of the spectrum.

Figure 2 depicts the spectra of extracts of the same cultures. In the upper left are the spectrograms of extracts of E. coli, staphylococci, and enterococci. Their characteristic bands lie within the limits from 6 to 10 microns and also differ from each other based on

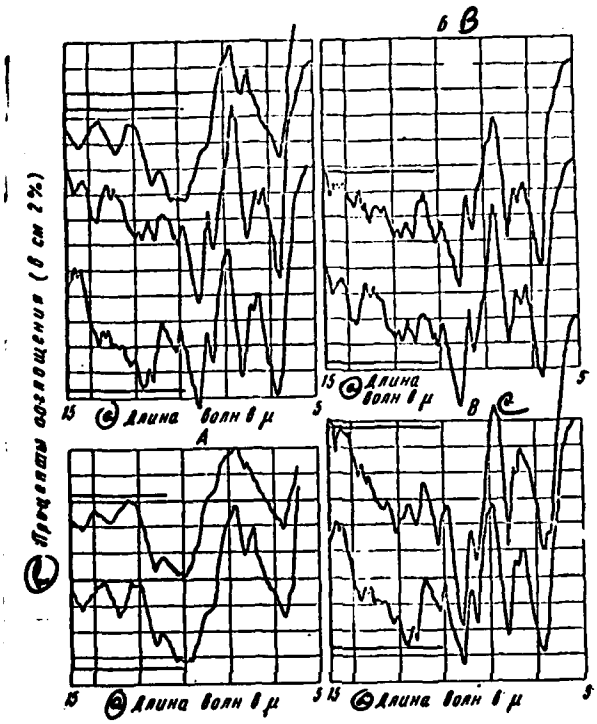

Fig. 2. Spectra of extracts of cultures: A - E. coli; B - staphylococci; C - enterococci.
Key: (a) Wavelength in microns; (b) Percentages of absorption (in cm 2%).

configuration of curves in this region. Individual spectrograms - A - E. coli, B - staphylococci, C - enterococci - show the recurrence of the recording for the same kind of extracts.

In contrast to the spectrograms of non-destroyed cells, in the extracts there are more varied bands of absorption. If a comparison is made of the resulting spectrograms of culture extracts with data from the literature, then here there are bands connected with polypeptide chains (6.4 microns), connected with nucleic acids (8--8.1 microns), and with phospholipids - 9.2 and 5.7 microns, which indicates an increased amount of fatty acids.

In comparing the spectrograms of non-destroyed microbial cells with the spectrograms from the investigation of the extracts, we came to the conclusion that though the first method was simpler and more

rapid, the second (method of extraction), being considerably more complex than the first, is more reliable and accurate and ensures a more thorough study of the fine structures of the microbial cells. The method of direct study of microbial cells may be used for obtaining rough data.

For laboratories in which the interpretation of the resulting spectrograms has to be carried out it is necessary to have a card index of standard coded charts in order to exclude the element of subjectivism in the evaluation. For sorting the cards it is best to use electromechanical and electronic equipment.

Thus the method of infrared spectroscopy is definitely promising, since it, in addition to giving completely objective data, ensures the quick determination of microbes. According to the data of Wilams (1956) and others, the entire process of determination of type of microorganism should occupy no more than 30 hours. And in actuality the setting up of the preparation from non-destroyed cultures and recording on the spectrogram require no more than 40 minutes. It is possible to obtain a spectrogram from culture extracts in 3--3½ hours. If in the course of investigation of objects of the external environment the method of membrane filters is used, then the time for obtaining the results of the seedings will fluctuate, depending on the type of microorganisms, from 10 to 18 hours. The inoculation of suspicious colonies on standard media along with their incubation will not exceed 6--9 hours, since for carrying out spectrophotometric analysis a small quantity of microorganisms is sufficient. Thus the entire process of indication can be handled in 30--35 hours.

Realization of the use of this method in practicing laboratories requires: 1) serial production of an infrared spectrophotometer of new simplified design, mainly for biologists, with one prism of NaCl; 2) creation of a reference library of standard spectrograms, for which it is necessary to have a more detailed study of as great a number as possible of strains of each species of microbes and their type variants.

What has been said are the first steps in the mastering of the method of infrared spectrophotometry. Basically the methodical working out of conditions for obtaining spectrograms has been carried out. But even with the more refined development of this new and promising express method for the determination of microbes in the external environment it is necessary, just as in any other express method, to combine it with the widely known classic methods of bacteriological investigations, which make it possible, though in longer periods, but with a higher degree of reliability, to establish the species affiliation of microorganisms.